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14. ABSTRACT Polycystic kidney disease (PKD), the most common life threatening genetic disease and affects approximately 1:400 people in the US including significant numbers of Active Duty Military personnel, Veterans and their beneficiaries. In PKD, the affected renal epithelia form cysts, eventually destroying renal architecture and function, leading to chronic kidney disease and end stage renal disease. There is currently no specific therapy for PKD. The present research examines the innovative translational therapy of intravenous administration of adult renal tubule cells containing the wild type (normal) PKD Pkhd1 gene in experimental PKD (the PCK rat model). The aim is to replace abnormal renal epithelia while avoiding the morbidity and mortality of surgery. Expansion of cells in vitro will also extend the utility of organs available for transplant. Data obtained to date demonstrate markedly lower renal cyst volume and fibrosis and better kidney function with cell transplantation. There are two potential mechanisms of action, either transfer of genetic material via exo-RNA or re-orientation of the cystogenic phenotype by correction of abnormalities in planar polarity. These results are the basis for an additional funding pre-application to extend the present studies, ultimately to prevent or treat kidney failure in humans. The results will also be presented at the Annual Meeting of the American Society of Nephrology in November, 2013.					
15. SUBJECT TERMS polycystic kidney diseases; renal insufficiency, chronic; kidney failure, chronic; exosomes					
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INTRODUCTION

Polycystic kidney disease (PKD) includes several monogenetic disorders typically caused by a mutated protein constituent of the primary cilium, a presumed sensory/signaling organelle. This defect typically results in severe renal cystic malformation and kidney failure. We successfully limited renal cyst formation with cell transplants and gene transfer in the PCK rat, a model of autosomal recessive PKD caused by a defect in the Pkhd1 gene.

BODY:

Task 1: Primary cell culture

1a. transfect cells to promote tubule formation

- A cells (control, transfected with an empty vector)
- B cells (transfected with a vector expressing serum amyloid A)

The primary renal cells were obtained from normal male Sprague Dawley (SD) rats. The detailed isolation and preparation methodology is described in the attached manuscript (Appendix). In brief, kidneys from normal adult male SD rats were digested with collagenase and the renal tubules were harvested, separated from glomeruli by differential sieving, and cultured. Renal cells then grew from the tubules and were transfected with either an empty plasmid vector (A cells or control cells) or with a plasmid vector designed to express Serum Amyloid A 1 protein (B Cells or experimental cells). After 5-7 days in culture the cells are expanded for direct cell transplantation or for production of exosomes used for treatment as well. The cells used for transplantation are usually $1-2 \times 10^6$ cells given intravenously in the tail vein as single doses. .

Task 2: Animal regulatory approvals were obtained from LARC and ACURO

Task 3: Animal surgery is performed in female PCK rats.

3a. The experimental model under general anesthesia. The procedure involves unilateral renal ischemia at 6 weeks of age (Ischemic group). This procedure requires clamping of the left renal pedicle for 50 minutes. In addition we separate groups of PCK rats are subjected to sham surgery. Sham surgery involved an identical operation except that the renal pedicle was not clamped (Sham group). The primary objective was to eliminate mutated cells and allow more space for the transplanted cells to engraft. The rats were subjected to dynamic contrast computed tomography of both kidneys to visualize and measure the renal cyst volumes. The rats were then terminated at 25 weeks of age.

3b. The modifying role of SD rat kidney cells in PCK rat kidney cell cyst formation: PCK rats and SD rats were sacrificed for renal cell isolation and culture to examine the direct and indirect role of SD rats renal cells on PCK rats renal cells cyst formation in vitro. This goal was accomplished by creating a model of PCK rat cell cyst formation using PCK rat renal cells embedded in Matrigel matrices. To test the direct role, SD kidney cells were cultured for 7 days and their exosomes harvested and added directly to PCK rat kidney cells in culture. The exosome treated and untreated PCK cells were then incorporated into matrigel matrices for 5

days and cyst number was counted in the gels. To test the direct role of SD cells, SD and PCK cells were combined in different proportions immediately before incorporation into matrigel matrices. The end-point was cyst number counted after 5 days in culture.

Task 4: Cell transplantation (three doses of either “A” or “B” cells are given intravenously via the tail vein).

4a. cell transplantation (1 day after surgery, 6 week old rats)

4b. cell transplantation (14 days after surgery, 8 week old rats)

4c. cell transplantation (28 days after surgery, 10 week old rats)

PCK rats were divided into six separate groups, Control (2 groups), Sham (2 groups), and Ischemic (2 groups):

1. Sham Control, did not receive cells,
2. Ischemic Control did not receive cells
3. Sham received 3 separate doses of A cells
4. Sham received 3 separate doses of B cells
5. Ischemic received 3 separate doses of A cells
6. Ischemic received 3 separate doses of B cells

Task 5: measurement of kidney function

5a. sampling of 50 ul blood at time 0 (surgery), daily for 1 week, then twice each month until termination at 25 week of age. This sample was used to measure serum creatinine.

5b. urine collection twice each month for measurements of protein and creatinine ratios

Task 6. Intravital imaging

6a. Three rats in each group were subjected to dynamic contrast computed tomography of both kidneys. This contrasted dynamic procedure yielded information on the renal function, GFR and blood flow. In addition static CT images were obtained to measure kidney and cyst volumes. The CT scans were obtained 2 weeks before termination.

Task 7. Processing fixed kidney tissue

7a. Section tissue.

Kidneys, livers, lungs and spleens from all animals were harvested at termination. The organs were fixed in 4 % paraformaldehyde and 100 uM sections obtained with a vibratome. The thick sections were examined under the fluorescent microscope to identify the location and number of transplanted cells in all the organs harvested. One half of the kidney was embedded in paraffin and thin sections were obtained for histology. One fourth of the kidney was frozen in liquid N₂ and used to extract DNA for genotype and for total RNA for specific mRNA determinations.

7b. probe tissue to measure cyst formation and size, fibrosis. Paraffin kidney and liver sections were stained with periodic acid Schiff (PAS) stain to visualize structure and able to measure cyst number and volumes. The sections were also stained with Masson's trichrome to visualize

and measure glomerular and interstitial fibrosis. The kidney sections were also stained with Leder's stain to visualize and count infiltrating leukocytes.

Task 8. Analysis of results

8a. Tabulation of results

8b. Statistical analysis

The documentation for task 8 regarding our results and statistical verification is included as part of the attached manuscript.

Task 9. Reporting of results

8a. Manuscript preparation/revision (appendix)

8b. Grant applications (appendix)

8c. Results today

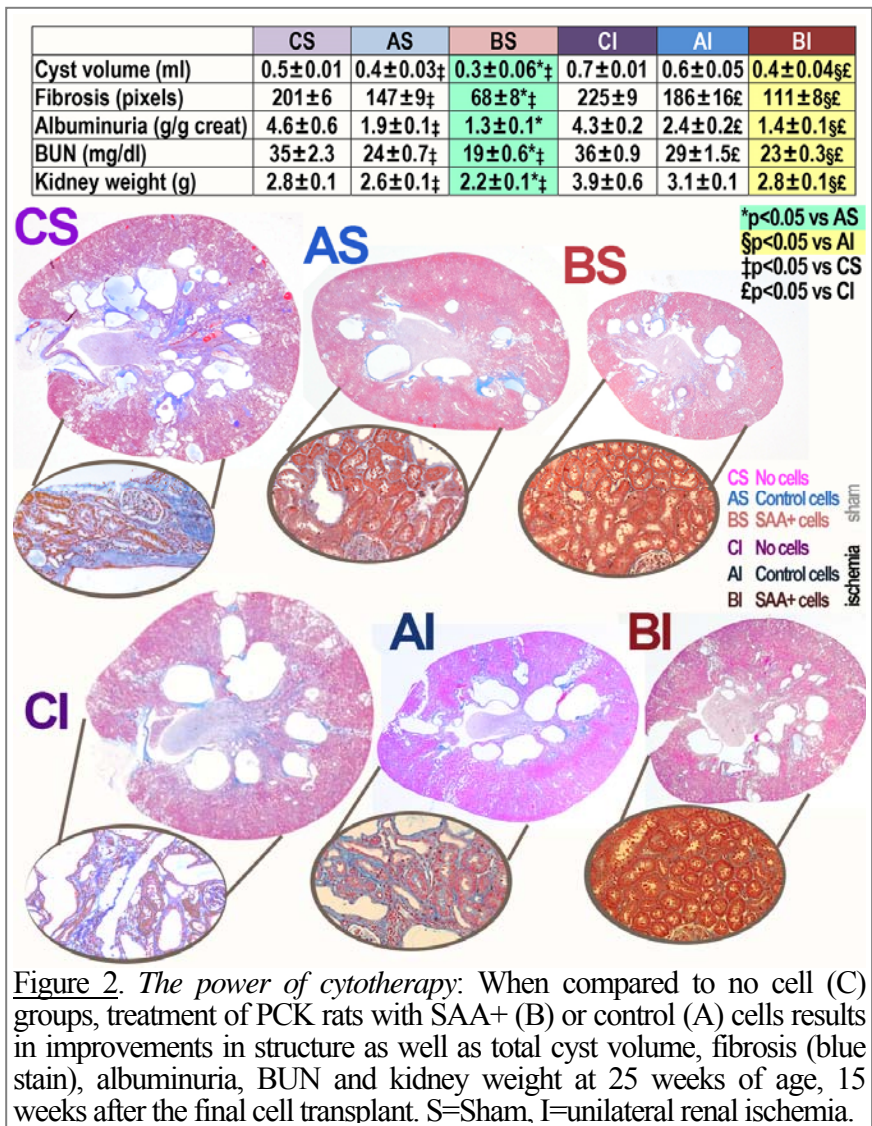
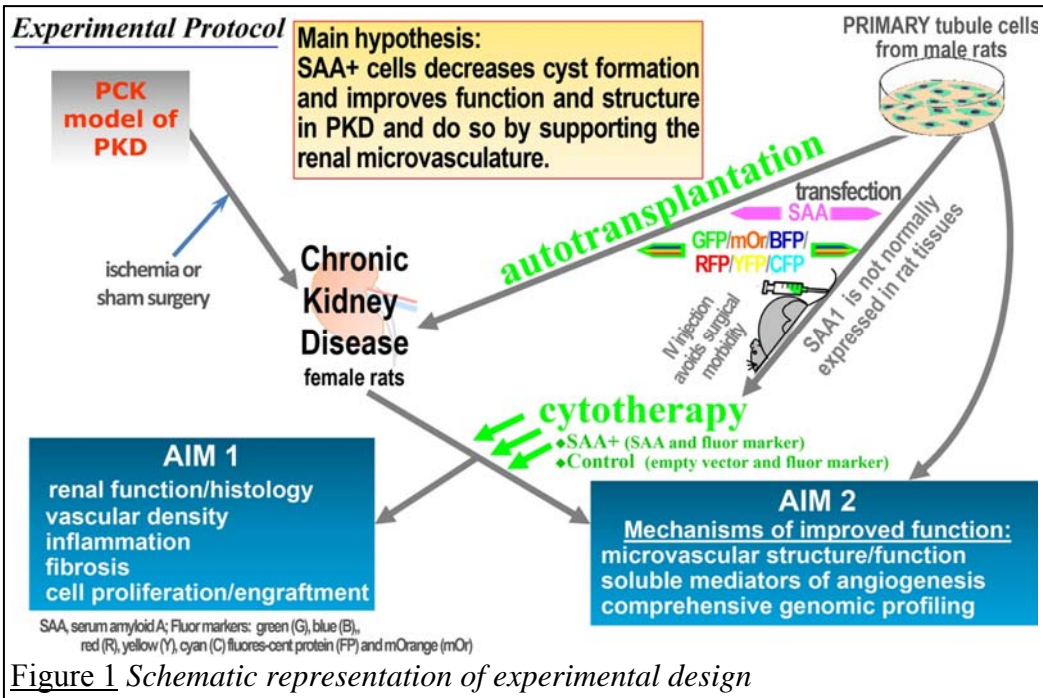
FINAL REPORT

PR110473 - Intravenous Renal Cell Transplantation for Polycystic Kidney Disease

Polycystic kidney disease (PKD) is one of the most common genetic diseases. Because PKD occurs in up to 1:400 births, PKD is a common cause chronic kidney disease (CKD) and of end-stage renal disease (ESRD) in military, Veterans, their beneficiaries and the general population (1). Perhaps more importantly, the transplantation techniques developed in these studies will be relevant to acute and chronic kidney disease of other etiologies which disproportionally affect Veterans.

Despite identification of the genetic abnormalities leading to PKD, and significant advances in understanding the pathophysiology of PKD, there is no effective therapy to prevent or limit progression of renal dysfunction to ESRD in PKD. The number of PKD ESRD patients may be increasing: USRDS 2013 (1) reports 28.2 (per million population) PKD patients on dialysis in 1985, 62.9 in 2000 and 92.5 in 2011. Although these data may reflect better diagnosis and reporting, they still illustrate that ESRD from PKD is a huge health problem. The main goal of this proposal is the development of effective cell and gene therapy protocols that prevent disease progression in PKD. A secondary aim is to understand the mechanisms of renal healing by cell transplantation, including reduction in cyst volume and renal scar formation. The results may also positively affect other forms of chronic kidney disease (CKD) and ischemic injury. The ultimate goal is prevention of ESRD in Veterans and others with kidney disease.

Dr. Dominguez and his team have developed, with current funding from the DoD and VA, novel cell transplant protocols that improve renal function and structure in multiple models of renal failure (2-5). The overall experimental design is presented in figure 1. Adult tubular cells are harvested from normal male SD rats. The kidneys are removed and tubules immediately separated by collagenase digestion and cultured in epithelial medium (4-5). The isolated tubules are then transfected with plasmid constructs designed to over-express the gene for Serum Amylase A1 protein (SAA) and also for green fluorescence protein (GFP). The latter serves to track the cells in vivo after the transplants. SAA is an acute phase reaction protein added for two main reasons, SAA induces a strong tubulogenic program in cultured renal epithelial cells and it also enhances their proliferation. We typically get much better results when the transplanted primary renal cells express SAA (4-5). After one week in vitro the cells were ready for transplantation via the tail vein of female rats; typically 2X10⁶ cells/rat.



PRELIMINARY DATA: CKD has reached epidemic proportions and results in immense suffering and loss of life, so the introduction of novel and effective therapies is urgently needed. We found that non-invasive renal cell transplantation improved survival and kidney function in diverse models of renal failure in rats (3-5). The transplant protocols have the advantage of using cells from one kidney to *rescue function in multiple* animals and are based in these preliminary observations:

- ◆ **The acute phase protein serum amyloid A (SAA) is critical in tubule formation** (Kelly KJ, Kluve-Beckerman B and Dominguez JH, Am J Physiol 296: F1355, 2009)
- ◆ **Non-invasive infusion of SAA expressing renal tubule cells significantly improves function and structure in established renal failure in multiple models** (Kelly KJ et al, Am J Physiol 299: F453, 2010 and Kelly KJ et al, Am J Physiol 303: F35, 2012), including: Acute kidney injury (AKI) due to ischemia, gentamicin or cisplatin-mediated injury. Chronic kidney disease secondary to diabetes, polycystic kidney disease or cisplatin. Auto-transplantation of cells harvested from an injured kidney rescues function in the *same animal* with CKD due to cisplatin (4). From this preparative work we documented that unlike stem cell protocols, male **donor SAA expressing cells labeled with green fluorescent protein (GFP) can be demonstrated in female recipient kidneys months after cell transplantation**. We have found that direct exposure of cultured renal tubular cells to SAA results in formation of functional tubules that transport the organic anion fluorescein and express characteristic tubule transporters (3,4). SAA is also expressed during tubule formation *in vivo*, in the mouse embryo and during renal regeneration after AKI, consistent with its critical role in tubulogenesis (3,4).

KEY RESEARCH ACCOMPLISHMENTS:

- To assess the ability of SAA expressing cells to support reconstitution of tubules *in vivo*, rats were given gentamicin, cisplatin or subjected to renal ischemia. After renal failure was established, 10^6 control (transfected with empty vector as well as green fluorescent protein, GFP) or SAA expressing (transfected with SAA and GFP) renal tubular cells were injected intravenously. **Mean serum creatinine was significantly better after treatment with SAA+ cells in each model** and infused GFP+ cells were found in recipient kidney tubules 1, 5 and 7 days after injection (4,5).
- These studies were then extended to primary cells to foster clinical applicability. Primary renal tubule cells transfected with SAA also rescue function in established AKI (4,5). Significantly better survival and histology was also seen in the groups that received SAA+ cells (4,5). **Treatment with SAA expressing cells also improves renal function and structure in other models of renal failure:** CKD due to cisplatin-mediated injury (4), diabetic nephropathy (Am J Physiol. Renal in press) and in PKD (figure 1).

- The current report is dedicated to research on the PCK rat model of adult recessive PKD (6). The PCK rat model has significant advantages: The mutated gene in the PCK rat (PKHD1 or fibrocystin) is orthologous to the human gene; the phenotype is very similar to the human phenotype in both autosomal dominant (ADPKD) and recessive (ARPKD)

PKD. Furthermore, cystic disease in the PCK rat, while extensive, allows time to intervene, whereas in other models, cystic disease progresses too fast for successful intervention and, in that respect, those super aggressive models differ from human PKD.

- The PCK rats were intervened at 8 weeks of age, one group was sham operated and the other was subjected to unilateral renal ischemia for 50 minutes. The purpose of the surgery was to eliminate as many genetically defective tubular cells –carry the mutated PKHD1 gene- as possible and make room for normal tubular cells. The rats were then transplanted, receiving cell infusions, either SAA- (A) or SAA + (B) cells, 2 days, 2 weeks and 4 weeks after surgery. The renal tubular cells were obtained from normal SD male rat donors, selected because of their related genetic background. The rats were terminated at age 25 weeks of age, or 13 weeks after the last cell transplant. There were two additional rat control groups that did not receive cell transplants. In summary the six PCK rat groups were:

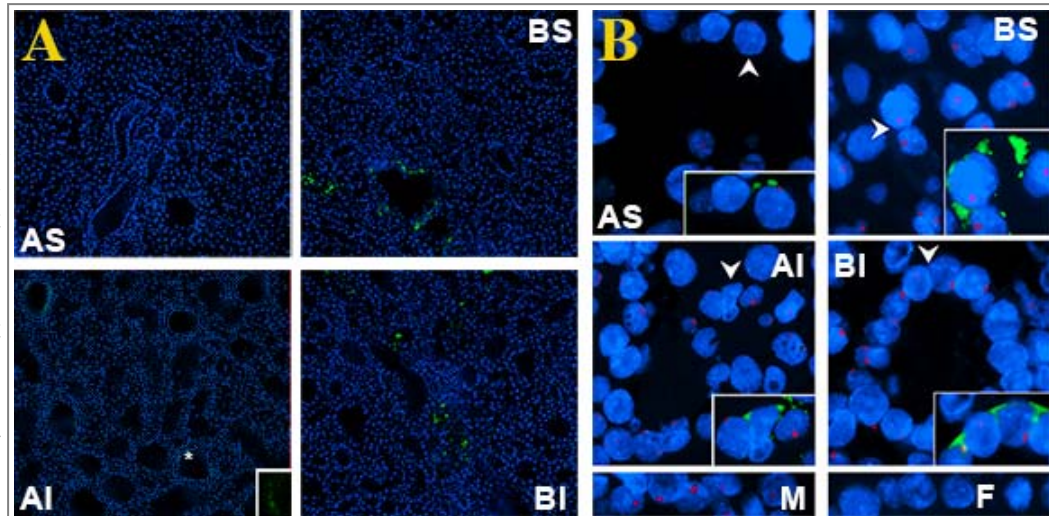


Figure 3. Engraftment of transplanted cells: PCK kidney sections at 25 wk of age show GFP+ cells in tubules and cysts (A). In AI, green fluorescence of the area at the asterisk is shown in the inset. Engraftment is also apparent as Y chromosome+ (red) in renal nuclei of females that received male cells (B). The insets show green fluorescence of areas at arrows, showing GFP and Y chromosome in the same cells. Nuclei are stained with DAPI (blue). AS, control cells/sham surgery; AI, control cells/ischemia; BS, SAA+ cells/sham; BI, SAA+ cells/ischemia; M, normal male; F, normal female.

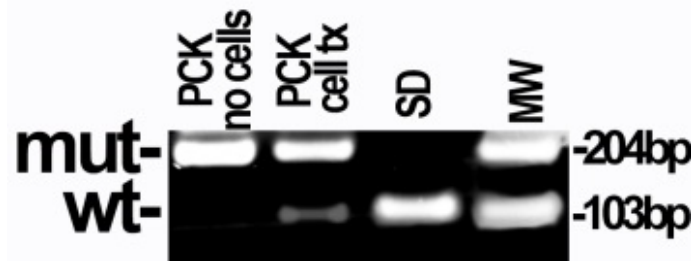


Figure 4. Genotype of the PCK mutation: PCR demonstrates the presence of both wild type (wt) and mutated (mut) PKHD1 genes in kidneys from rats that received SAA+ cells but not in untreated PCK or normal Sprague Dawley (SD) rats. PCR genotype was performed using primers specified by Charles River:

Mut-F: 5-AAG CCA AAT CTT TCT CTT TTC CT-3'

Mut-R: 5'-CTT GCT GTC CGA ATA CCA C-3'

Wt-F: 5'-ACT GCC TTT TAC TGA AGC ATT TAA C-3'

Wt-R: 5'-TGG AAG GAA AAG TTG CCC T-3'

MW, molecular weight markers

1. Sham operated rats receiving SAA- (AS) cells (n= 5)
2. Sham operated rats receiving SAA+ (BS) cells (n=5)
3. Unilateral renal ischemia rats receiving SAA- (AI) cells (n= 5)
4. Unilateral renal ischemia rats receiving SAA+ (BI) cells (n= 5)
5. Control sham operated rats, No cells (n= 5)
6. Unilateral renal ischemia rats, No cells (n= 5)

Although the mechanism of renal failure in PKD is distinct from other forms of CKD, we hypothesized that cytotherapy with renal cells from male SD rats containing the normal PKHD1 gene would improve structure and decrease cystogenesis in PKD. The rats survived with the exception of two rats in the AI group, which died two weeks after the second infusion. The remaining PCK rats were terminated at 25 weeks of age (15 weeks after the final cell dose), **total cyst volume, renal fibrosis (quantified on trichrome stained sections), albuminuria, blood urea nitrogen (BUN) and kidney weight were**

significantly decreased in groups treated with SAA+ cells as compared to those groups that received SAA- control cells (figure 2).

Unlike stem cell protocols, infused cells are found in kidneys weeks

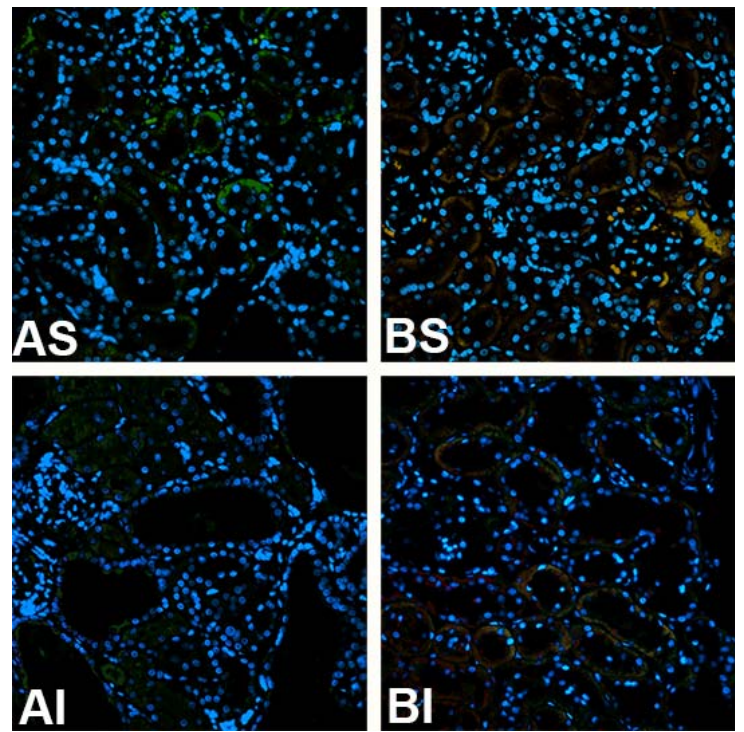


Figure 5. Expression of SAA1 co-localizes with GFP in PCK rats given SAA+ cells: Representative images from kidneys stained for SAA (red) show GFP+ cells in kidneys from rats that received control (A) cells. SAA co-localizes with GFP (resulting in orange) in the kidneys from the groups that received SAA+ cells. All nuclei are labeled with DAPI (blue). AS, control cells/sham surgery; AI, control cells/ischemia; BS, SAA+ cells/sham surgery; BI, SAA+ cells/ischemia

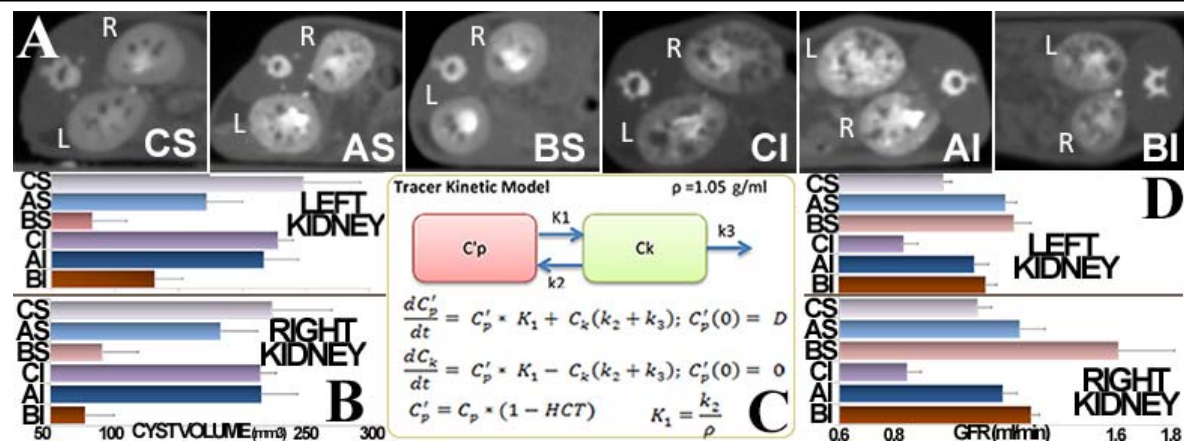


Figure 6. Dynamic contrast computed tomography imaging of decreased cyst volume with cytotherapy: Representative CT images of kidneys from 25 wk old PCK rats treated with control cells, SAA+ cells or no cells following sham surgery or unilateral renal ischemia (A). Graphs of preliminary data in 17 rats show improvement in cyst volume (B) and GFR (D) with SAA+ cells. In addition, control cells result in improvement when compared to the “no cell” groups. The 2 pool model for determination of GFR is shown in C. C, concentration; p, plasma; k, kidney; K, constant; t, time; hct, hematocrit; ρ , density of contrast media (1.05g/ml); CS, no cells/sham surgery; CI, no cells/ischemia; AS, control cells/sham surgery; AI, control cells/ischemia; BS, SAA+ cells/sham surgery; BI, SAA+ cells/ischemia

after the last cell transplant (our Ref 4, 5 and also figure 3). In addition to identifying the GFP+ cells, continuous engraftment is documented using fluorescence in situ hybridization (FISH) for the Y chromosome (red) in kidneys from female rats that received male cells (figure 3). Moreover, PCR shows both the normal and mutated PKHD1 gene (figure 4) and the presence of the male determining SRY gene in kidneys from rats that received SAA+ cells (not shown). SAA protein can also be demonstrated in groups that received SAA+ cells, figure 5.

To determine the tubule segments from which cysts originate, kidneys from PCK rats were labeled with lectins *dolichos biflorus* (conjugated to rhodamine) and *lotus tetragonolobus* (pseudo-colored cyan) to label collecting ducts and proximal tubules, respectively. Cystic dilation was seen largely in collecting ducts with some cysts emerging from proximal and unlabeled tubules. GFP+ (transplanted) cells were found in both proximal and collecting duct cysts (not shown).

We have also examined the severity of cystic disease and split renal function by dynamic CT (figure 6). In preliminary results obtained 2 weeks before termination, total cyst volume was less and renal function (glomerular filtration rate, GFR) better in rats treated with SAA+ cells.

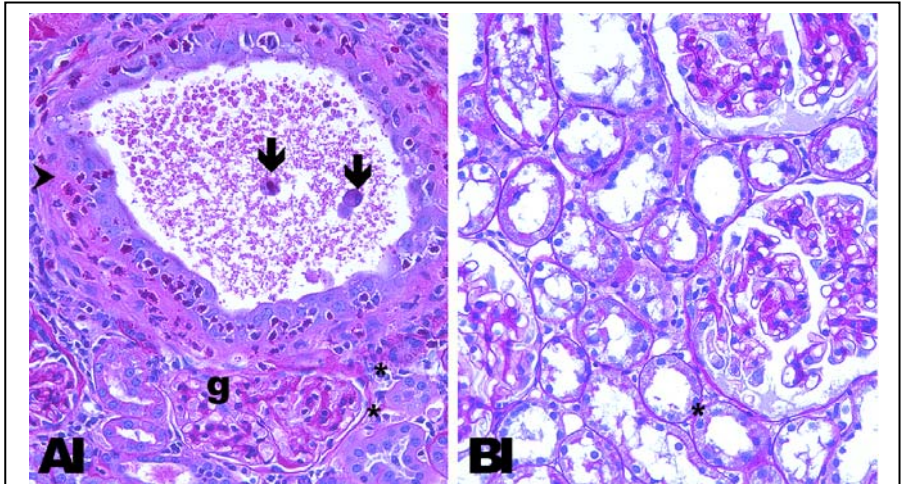


Figure 7. *Post-ischemia PCK kidneys:* Representative PAS stained kidney sections from 25 wk old PCK rats post-ischemia at 6 wk of age and cell transplanted three times with SAA negative cells (AI) or SAA+ cells (BI) are shown. In AI, the expanding renal cyst contains leukocytes (arrows) and crushes a glomerulus (g). The arrow head points to the fibrotic cyst capsule. The asterisks are in peritubular spaces filled with leukocytes. BI only shows microcysts, glomerular structure is grossly preserved, and peritubular spaces are devoid of inflammatory cells.

- HYPOTHESIS:** Data from others and our own data (from work conducted for the Department of Defense, Discovery Award PR110473) guided us to propose the following testable hypothesis: **“intravenous transplantation of rat adult kidney cells, expanded and re-programmed in culture, limits cyst formation and improves structure and function in polycystic kidney disease”**. Accordingly, the implicit mechanisms of cell action are based on limiting cyst growth. The assumption is that cyst growth is a critical determinant of progression at two fundamental levels: 1) Cyst expansion compresses and damages surrounding functional tissue rendering it dysfunctional. In figure 7 we illustrate this point showing the compressing effect of an expanding cyst on a glomerulus in its vicinity. 2) Cyst expansion demands dedicated blood supply, recruiting enough blood vessels to form dense vascular

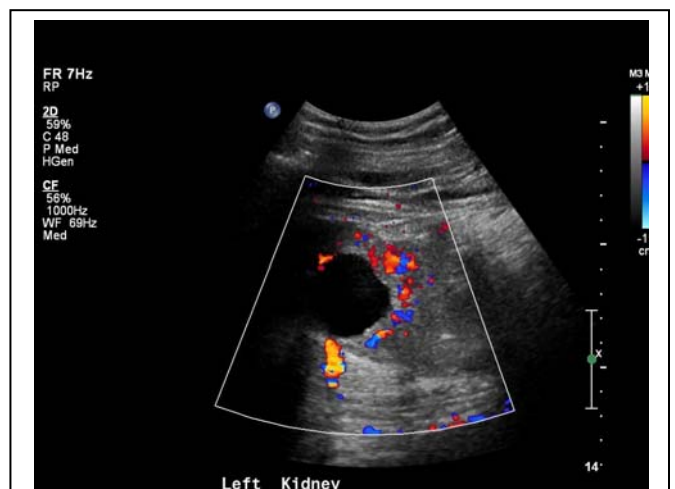


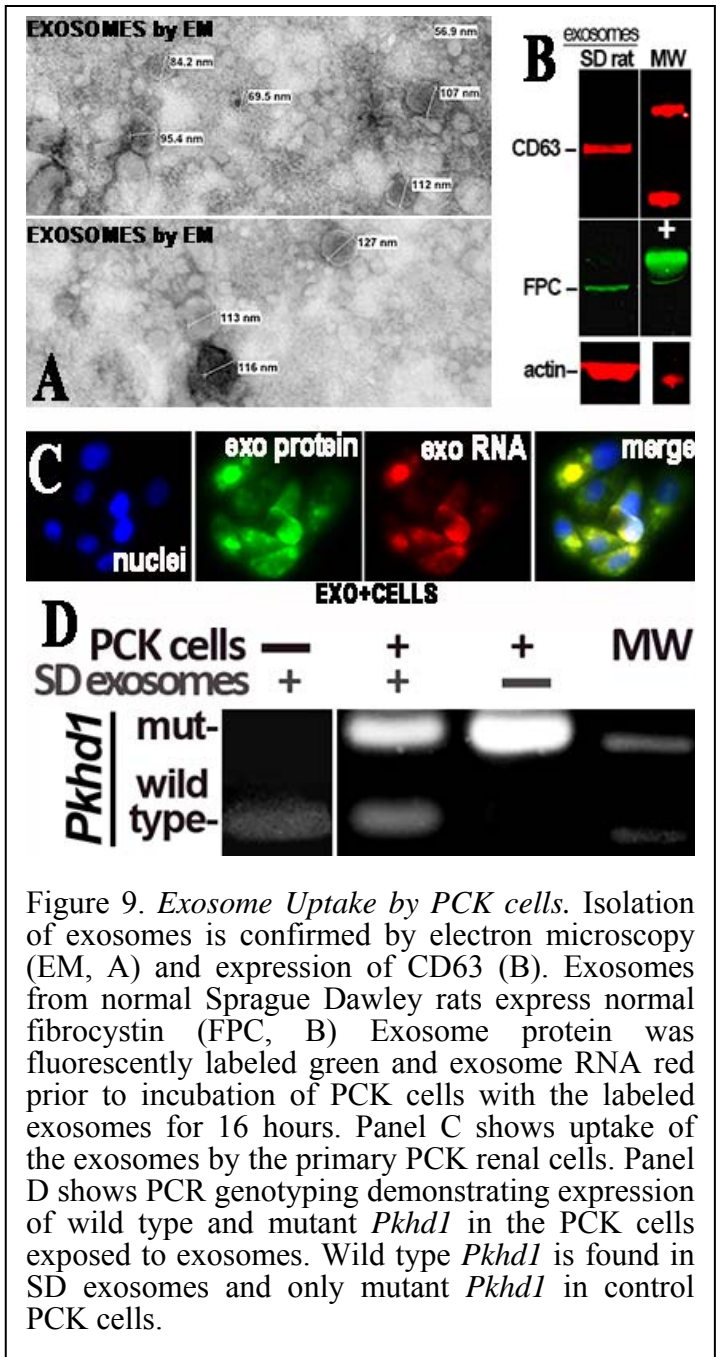
Figure 8. Renal Doppler/US: Vascular capsule around a cyst in human PKD.

capsules around the emerging cysts, figure 8. One consequence of the vascular sequestration is a limited and sparse peritubular vasculature in functional tubules. We presume that these thin vascular networks are inadequate and enhance tubular susceptibility to ischemic injury, a pro-cystogenic stimulus.

CURRENT AND FUTURE WORK: Our ongoing and projected future work on PKD will be focused on one major question emerging from our past research. We found that a comparatively (with respect to the host kidneys) small number of anchored transplanted cells had major beneficial effects on the entire kidney morphology and function. The transplanted cells were properly anchored in renal tubules and remained in the kidney for weeks post-infusion. Therefore, we have considered two main possibilities that might be working together and explain the amplified response to cell transplants.

First, we studied and demonstrated that harvested SD rat renal tubule exosomes contained mRNA (exo-mRNA) encoding the wild type (normal) *Pkhd1* gene. We then found that renal tubule cells from PCK rats were able to internalize and express the *Pkhd1* exo-mRNA after exposure to SD rat renal exosomes in culture, figure 9. Furthermore, those PCK renal cells exposed to SD renal exosomes were much less able to form cysts when incorporated into matrigel matrices. Supporting the idea that introduction of a normal wild type *Pkhd1* gene drastically limited PCK cell cystogenesis in matrigel matrices. Hence, it is possible that exo-mRNA released by transplanted cells limited cystogenesis in the host PCK kidneys.

Second, normal SD rat renal cells when combined with PCK rat renal cells limited subsequent PCK cell cystogenesis in matrigel matrices. It is noteworthy that adding as little as 2 % SD cells drastically limited cystogenesis in 98 % PCK cells. This striking effect was dose-dependent and at 20 % cells combined with 80 % PCK cells, cystogenesis was nearly abrogated, figure 10. This finding is detected as early as 2 days in culture, which points towards an organization event of renal PCK cells directed by a minority of renal SD cells. We surmise that a few SD cells can re-orient the majority of renal PCK cells from a cystic phenotype



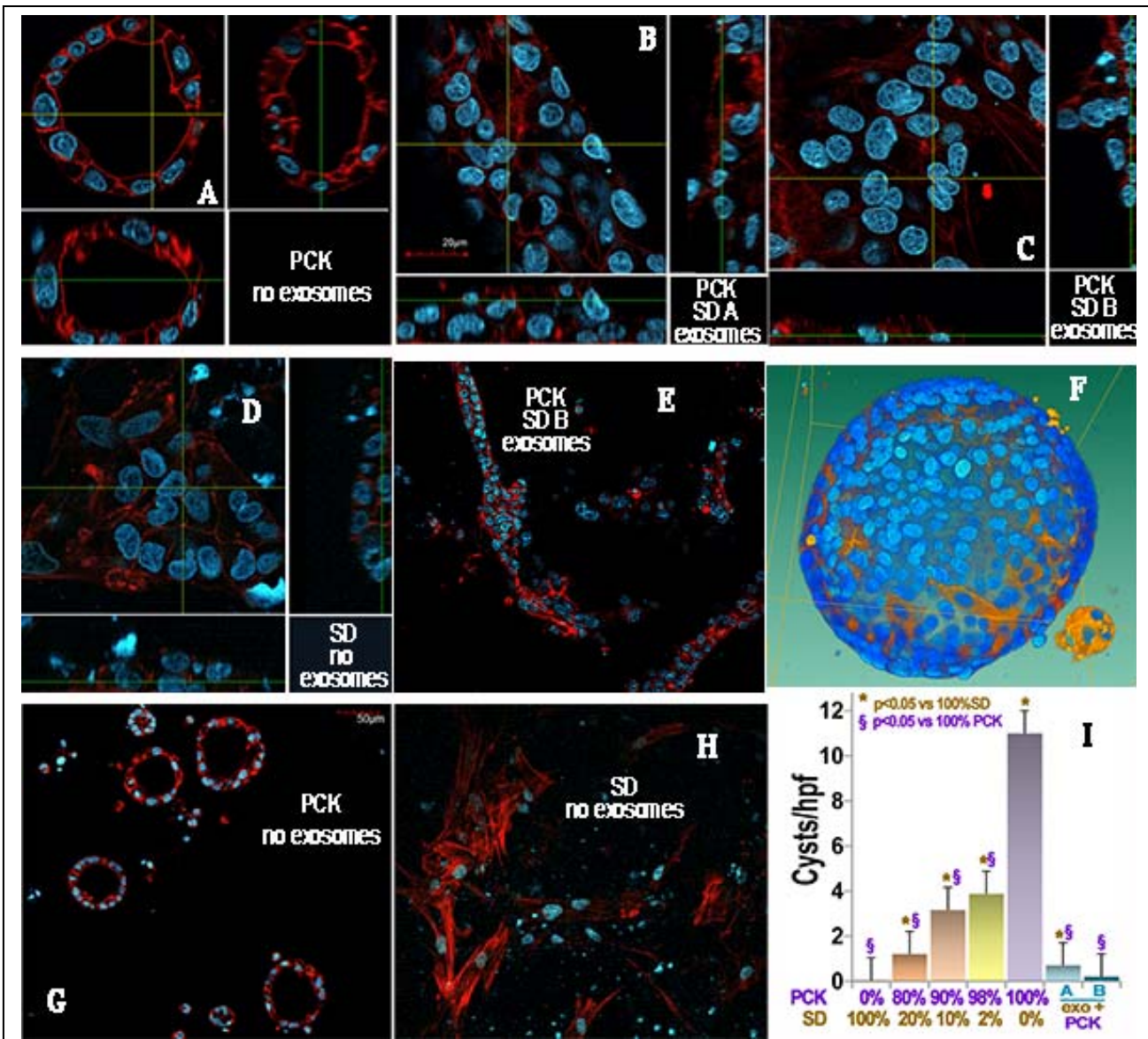


Figure 10. *The effect of exosomes on PCK cysts in culture.* Representative confocal images of cells stained with rhodamine phalloidin to label actin red and Hoescht to label nuclei blue demonstrate cysts in renal cells from PCK rats grown in matrigel matrices without exosome treatment (A, F, G). Neither PCK cells cultured with renal exosomes (B, C, E) from Sprague Dawley (SD) rats nor SD tubular cells (D, H) form cysts in matrigel. The higher power images (A-D) show orthogonal projections to demonstrate the clear presence or absence of cysts. Panel F shows a 3D reconstruction of multiple images demonstrating the cystic nature of the structures formed by PCK cells. The lower power images (E, G, H) demonstrate tubular structures formed by PCK cells previously exposed to exosomes, multiple cysts in one field of PCK cells and the absence of cysts in SD cells, respectively. Quantification of cyst number in a total of 168 fields is presented in the graph (I). B exosomes are from SAA+ cells, A are from SAA- control cells. Both A and B renal cell exosomes contain wild type *Pkhd1* (above).

to a tubulogenic phenotype. It is possible that the effects of the abnormal planar polarity of PCK cells were diminished by the normal planar polarity of the SD cells. This effect resulted in the more desirable tubulogenic phenotype. In short, transplanted normal cells will mainly reside in developing micro cysts and limit their expansion by correcting the polarity of adjacent epithelial cells. The new hybrid tubules may recruit more blood vessels and improve delivery of oxygen and nutrients.

REPORTABLE OUTCOMES: American Society of Nephrology annual meeting invited presentation November 2013.

Additional DOD research funding pre-application submitted to extend these studies, ultimately to humans (June 2014).

CONCLUSIONS polycystic kidney disease and the resultant renal failure result in tremendous suffering and loss of life in Veterans and the general population and the novel, non-invasive cytotherapy protocols reported here have tremendous potential to help large numbers of patients with CKD and ESRD. Our renal regeneration protocols also have the potential to limit the progression of CKD and even restore function when patients reach the point of ESRD.

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APPENDICES: MANUSCRIPT

SUPPORTING DATA: Figures embedded in the main text

Novel Means of Gene Therapy in Experimental Polycystic Kidney Disease

Katherine J. Kelly¹, Jizhong Zhang¹, Ling Han¹, Malgorzata Kamocka¹, Caroline Miller²,
Vincent H. Gattone II^{2,4} and Jesus H. Dominguez^{1,3}

In Memory of Vincent H. Gattone II, PhD

From the Departments of Medicine (1) and Anatomy (2), Indiana University School of
Medicine and Nephrology Division, Veterans Administration Medical Center (3), Indianapolis
IN; deceased (4)

Abbreviated title: Gene therapy via cytotherapy with tubular cells in PKD

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Conflicts of Interest: none

ABSTRACT

Polycystic kidney diseases (PKD) are the most common life-threatening genetic diseases, and can cause end stage renal disease (ESRD) with resulting suffering, mortality and cost. Despite of advances in understanding their pathophysiology, PKD remain incurable. Autosomal recessive PKD (ARPKD) is truly catastrophic, causing death and ESRD in neonates and children. Using PCK female rats, an orthologous model of ARPKD harboring mutant *Pkhd1*, we tested whether intravenous renal cell transplantation (IRCT) with normal Sprague Dawley (SD) male kidney cells reversed ARPKD. IRCT markedly reduced cyst volume, renal fibrosis, albuminuria, blood urea nitrogen and kidney weights in treated rats, as compared to PCK rats that received no cells. IRCT with cells containing serum amyloid A1 (SAA) and wild type *Pkhd1* had powerful and sustained beneficial effects on renal function and structure. In treated kidneys, donor cell engraftment and both mutant and wild type genes were found 15 weeks after the final cell infusion. To examine the mechanisms of protection, exosomes derived from SD cells were found to contain and transfer wild type *Pkhd1* to PCK cells. The results indicate that IRCT with SAA+ cells is safe and effective in delivering the normal gene and preventing progressive cyst enlargement and renal dysfunction in PKD.

INTRODUCTION

Most patients with autosomal recessive polycystic kidney disease (ARPKD) who survive the neonatal period suffer from severe renal complications early in childhood (1, 2). Genetic intervention, elegant and simple in theory, is the definitive treatment of genetic renal cystic disease. However, there are currently no safe and efficient clinical modes of gene transfer (3). In addition, an administered gene load must be precisely controlled. Abnormally low or high gene expression can cause cyst formation (4, 5). Hence, we tried a novel therapeutic strategy employing adult kidney cell transplantation, previously successful in other renal failure models (6-9). The earlier data include successful long-term kidney cell engraftment and renal regeneration in diabetic nephropathy and also cell auto-transplants (9). We used adult primary kidney cells reprogrammed (via a non-viral vector) to express the tubulogenic protein Serum Amyloid A1 (SAA,(6)). We now report that allogeneic adult kidney cells, given intravenously, improve renal structure and function in the PCK rat, which expresses mutant *Pkhd1*, orthologous to the human ARPKD mutation. Engrafted donor cells were identified in recipient kidneys 15 weeks after the last cell dose. We also tested the hypothesis that exosomes from transplanted cells positively affect PCK cells via transfer of genetic material. We propose that intravenous renal cell transplantation (IRCT) is a safe and very effective means to deliver the wild type *Pkhd1* gene and, most importantly, prevent progressive CKD in PKD. IRCT has the advantages of using cells from one rat to transplant multiple animals, non-invasive administration and avoiding immunosuppression.

RESULTS

Six groups of female rats with PKD were evaluated: three were subjected to sham surgery and three to unilateral renal ischemia at 6 weeks of age. We hypothesized that severe ischemia, common clinically in PKD, would reduce the number of mutant cells and facilitate engraftment of transplanted cells. PCK rats received renal tubular cell transplants from normal male Sprague Dawley rats intravenously when 6, 8 and 10 weeks old and were terminated at 25 weeks of age. Sham surgery rats were thrice infused with SAA negative (control “A”) cells (AS) or with SAA expressing (SAA+ “B”) cells (BS) or with no cells (CS). Postischemic rats also received 3 doses of SAA negative control (AI) or SAA+ (BI) or no cells (CI). A and B cells also expressed green fluorescent protein (GFP). One CI rat died when 23 weeks old. During the study, there were no significant differences in animal weights or final liver weights.

In figure 1 is shown the marked improvement in total cyst volume in sham and postischemic PCK rats transplanted with control A cells (containing wild type *Pkhd1*) and an even greater positive effect in groups that received B cells (containing both SAA1 and wild type *Pkhd1*) when compared to the groups (CS and CI) that did not receive cells. In addition to improved cyst volume and kidney weight, better function was observed in the B than A and C groups as shown by decreased albuminuria and blood urea nitrogen (BUN) (table 1) as well as decreased renal fibrosis, quantified as fractional area of collagen staining in trichrome stained sections (figure 2).

We hypothesized that expression of wild type *Pkhd1* occurred with IRCT. In contrast to stem cell protocols, we have clearly documented engraftment of donor cells months after transplantation in renal failure models (7-9). In this study, engraftment was demonstrated by multiple and independent techniques (figure 3): (1) fluorescence in situ hybridization (FISH)

showed the Y chromosome in female recipient kidneys transplanted with male cells, but not in normal females; (2) genotyping demonstrated both mutated and wild type *Pkhd1* in transplanted rats, but not in those that did not receive cells; PCR detected both (3) the male determining SRY gene in female kidneys transplanted with male cells but not in control females and (4) SAA mRNA in kidneys that received B (SAA+) but not A (SAA-) cells or in rats not given cells; (5) fluorescence microscopy showed GFP+ cells in kidneys of rats that received GFP+ A or B cells and (6) co-localization of immunoreactive SAA with GFP in kidneys from rats that received SAA+ B cells. In summary, renal genotyping demonstrated the effectiveness of cell transplantation as a means of delivering normal genes, in other words “gene therapy.”

Potential mechanistic pathway of improvement in renal structure and function are multiple. We have postulated that the broad benefit seen with cell transplants (7-9) points to a general action explained by improved vasculature with better delivery of oxygen and nutrients. It is known that major renal microvascular abnormalities aggravate human PKD, promoting renal dysfunction and cyst enlargement (10). Thus, the renal microvasculature was labeled with an anti-CD31 antibody to evaluate the role of cell transplantation, figure 4. Representative images illustrate severe glomerular microvascular attenuation in control PCK rats and in those transplanted with SAA- cells. In contrast, glomerular vessels were much better preserved in the groups that received SAA+ cells. Pericycystic hypervascularity, thought to contribute to cyst growth in human PKD (11), was markedly attenuated in cell treated rats. Given that the transplanted cells comprised only a small proportion of cells in the PCK kidneys, we also hypothesized that exosomes from transplanted cells (carrying wild type *Pkhd1*) transferred genetic material to PCK cells with mutant *Pkhd1*. To test this hypothesis, we isolated exosomes from normal SD rat renal tubular cells and verified

exosome isolation via electron microscopy and expression of CD63. The exosomes also expressed the protein product of *Pkhd1*, fibrocystin. Intra-exosome RNA and protein were labeled red and green, respectively. Labeled exosomes were taken up by cultured renal tubular cells from PCK rats, resulting in expression of wild type *Pkhd1* in cells incubated with exosomes from SD cells but not untreated PCK cells (figure 5). When the PCK cells were incubated in extracellular matrix (matrigel), 3D cystic structures were formed. When PCK cells were incubated with exosomes from SD cells, the cells remained non-cystic with occasional “tube” formation (figure 6). This result supports the hypothesis that exosomes derived from normal cells transfer genetic material and improve the phenotype in PCK cells.

DISCUSSION

The number of PKD end stage renal disease (ESRD) patients is increasing: US Renal Data System (USRDS) 2013 (12) reports 28.2 (per million population) PKD patients on dialysis in 1985, 62.9 in 2000 and 92.5 in 2011. While these data may reflect better diagnosis and reporting, they still illustrate that PKD is a huge health problem. Although the mechanism of renal failure in PKD is distinct from other forms of chronic kidney disease (CKD), we hypothesized that cytotherapy with cells containing the wild type *Pkhd1* gene would result in renal chimeras and improve structure and decrease cystogenesis in PKD. The PCK rat was studied because the mutated gene in the PCK rat (*Pkhd1*) is orthologous to the human gene; and the phenotype is very similar to the human phenotype in both ARPKD and autosomal dominant PKD (ADPKD). We suggest that IRCT can be applied to limit untreatable PKD. Furthermore, multiple patients can potentially receive cells from a single donor, a critical point since many ESRD patients never get renal transplants due to shortage of organs for donation (13).

The mechanism of action of SAA, an “acute phase reaction” protein, is unknown. SAA has strong tubulogenic properties (6) and while it is not expressed in the normal adult, it is expressed during tubulogenesis in the embryo and during repair after renal injury (6). In fact, even poorly differentiated rat NRK52E cells dramatically improve renal function in multiple acute kidney injury (AKI) models when re-programmed to express SAA (7). Studies with primary renal epithelial cells, to extend clinical applicability, also resulted in better renal regeneration in both AKI and CKD models (9). In addition, SAA+ renal tubular cells can rescue renal function in the same animal in an auto-transplant procedure (9). Improvement in renal function and structure were also observed with SAA+ cell transplantation in diabetic CKD (8).

The present results corroborate the great potential of primary renal cell transplants. In addition to providing the wild type *Pkhd1* gene, anchored donor tubular cells may positively influence recipient renal cells (for example via transferred fibrocystin or secreted vascular endothelial growth factor (9)). Thus, we have also shown that exosomes from normal SD cells contain wild type *Pkhd1* and its protein product, fibrocystin and that incubation of PCK cells with SD exosomes results in transfer of wild type *Pkhd1* to the PCK cells. In contrast to our cell transplant protocols, stem cells have not been shown to become functional renal cells (14-17) and, in some cases, the transplanted stem cells acquire a totally undesirable and uncontrolled phenotype in recipient CKD kidneys (18). In the present study, the broad benefit of cell transplants points to a general action which may be due to delivery of the wild type *Pkhd1* gene as well as improved renal vasculature and better delivery of oxygen/nutrients. In conclusion, we suggest that, in PKD, adult renal epithelial cells and exosomes offer a physiological and effective means to deliver normal genes and effect preservation of renal structure and function and limit cyst formation and expansion.

METHODS

Primary Renal Tubular Cells: Primary renal tubular cells were obtained from age matched male Sprague Dawley (SD) rats (Harlan, Indianapolis), and renal cells from one SD rat were equally distributed to four PCK female rats, one in each of the four groups described below. The SD rats were sacrificed by removing both kidneys under general anesthesia. The kidney cortices were minced in S1 medium (below), and digested with type 4 collagenase (Worthington, Lakewood, NJ), 6 mg/dl, at 37°C in 38 % O₂ and 5 % CO₂ for 50 minutes. Renal tubules were then separated by percoll gradient (9), divided into two sets, re-suspended in 300 ul of transfection buffer (HEPES, 20 mM; KCl, 142 mM; Dextrose, 6 mM; Na₂HPO₄, 0.7 mM; MgATP, 5 mM; and EGTA, 10 uM), and transfected by electroporation (40 Volts x 12 msec x 500 msec x 6 pulses). Control or SAA negative (group “A”) tubules were co-transfected with empty vector pcDNA3.1 (30 ug), pAcGFP1-C1 (15 ug, GFP is the cytosolic label used to track cells in vivo, Clontech, Mountain View, CA), and pCruz HASIRT1 (15 ug, Addgene), which expresses SIRT1, a NAD(+)-dependent deacetylase that may have a role in resistance to cellular stress and, thus, has been used in prior transplant protocols (9). For SAA1 positive (group “B”) cells pcDNA3.1 was replaced with pcDNA3.1-SAA1 plasmid, 30 ug, manufactured and sequenced in our laboratory as previously reported (6, 9). Transfection efficiencies were >70% (9).

The co-transfected tubules were cultured in S1 medium: Each 2 liter contained Ham's F-12, 10.7 gm; DMEM, 8.32 gm; L-glutamine, 0.29 gm; HEPES, 4.78 gm; sodium selenite, 1.7 ug; sodium pyruvate, 0.11 gm; phenol red, 3.2 ml and pH was adjusted to 7.4 with sodium bicarbonate (Sigma). S1 medium was supplemented with hepatocyte growth factor, 200 ng/ml, and epidermal growth factor, 400 ng/ml (R&D Systems, Minneapolis, MN). The medium also contained hydrocortisone 100 ug/ml; insulin, 35 ug/ml; transferrin,

32 ug/ml; sodium selenite 42 ng/ml (Sigma, St. Louis MO); with 20 % fetal calf serum; and G418, 75 ug/ml, was added after 48 hours of culture for selection. In preparation for transplantation, male renal tubular cells were lightly trypsinized after 7-8 days in culture, washed in PBS, and 10^6 cells injected intravenously in the tail vein of PCK female rats at 6 (2 days after surgery, below), 8 and 10 weeks of age.

Animal Protocols. All experiments were conducted in conformity with the "Guiding Principles for Research Involving Animals and Human Beings," and the protocol approved by the Institutional Animal Care and Use Committee. Female PCK rats (Charles River, Wilmington, MA) were acquired at 5 weeks of age and underwent left renal ischemia or sham surgery at 6 weeks of age. Anesthesia was accomplished with inhaled isoflurane (0.5-1%) prior to occlusion of the left renal pedicle for 50 minutes. Sham surgery consisted of an identical procedure except the renal pedicle was not clamped (19). The rats were later infused with donor cells: one sham group (AS, n=4) received SAA- (A) cells and another sham group (BS, n=4) received SAA+ (B) cells. One postischemia group (AI, n=5) received SAA- (A) cells and one postischemia group (BI, n=5) received SAA+ (B) cells. Control sham (CS, n=5) and postischemic (CI, n=5) rats did not receive cells. Weights, sera and urine were collected biweekly and chemistries were measured by the Indianapolis VA clinical laboratory. Urine protein was measured via ELISA according to the manufacturer's protocol (Exocell, Philadelphia, PA). Cystic change was quantified using point count sterology as described (20). Dynamic contrast computed tomography was performed on anesthetized animals 1-2 weeks before sacrifice using a high speed CT scanner as described (21).

Histology and immunohistochemistry: Kidney sections were fixed in 3.8% paraformaldehyde, paraffin embedded and 5 µM sections obtained for Masson's trichrome to stain collagen blue and periodic acid Schiff (PAS) to evaluate morphology. The areas of glomerular and peritubular fibrosis were quantified in blinded sections and expressed as fractional areas, covering all available sections. Additional kidney sections were immunostained with anti-CD31 (PECAM) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and Texas-Red conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA), to visualize the microvasculature. Paraformaldehyde fixed 100-µm kidney sections (Vibratome, St. Louis, MO) were immunostained with rabbit primary anti-SAA antibody (7, 9) and Texas Red conjugated secondary antibody. Nuclei were labeled with DAPI (Molecular Probes, Eugene, OR). Fluorescence images of CD31 and SAA staining and expressed GFP were collected with a Leica DMI 3000B fluorescence microscope. Confocal images were obtained with an Olympus FV1000-MPE microscope. Quantification of immunostaining was performed using Metamorph software in blinded sections.

Fluorescent in situ hybridization (FISH) of the Y chromosome: FISH was used to localize the Y chromosome in female kidneys 15 weeks after IRCT with male renal cells as previously reported (9) employing the fluorescent labeled rat Y chromosome probe (Rat IdetetTM Chr Y Paint probe red, IDTM556, ID Labs Biotechnology Inc. London ON, Canada). Sections were counterstained with DAPI prior to imaging with Leica DMI 3000B fluorescence microscope.

Renal SAA1 mRNA: RT-PCR was utilized for amplification of SAA1 mRNA in recipient kidneys at termination. RNA was purified from homogenized renal cortices as recommended

by vendor (Invitrogen, Grand Island, NY), cleaned with an RNeasy Mini kit as recommended by vendor (Qiagen, Valencia CA) and used to synthesize cDNA (AffinityScript QPCR cDNA Synthesis Kit, Aligent Technologies, Santa Clara CA). The murine SAA1 mRNA was amplified using PCR System 2400 (Perkin Elmer, San Jose, CA) with the following primers (6):

Forward 1: 5'-CGCCACCATGGAGGGTTTTTTTCATTTGTTTAC-3'

Forward 2: 5'-TACAGGCTAGCGCCACCATGGAGGGTTT-3'

Reverse 1/2: 5'TCAGGTGGATCCCTCAGTATTTGTCAG-3'

Identification of DNA encoding the male sex-determining region on chromosome Y (SRY) localized in female kidneys (8): DNA was extracted from the recipient kidneys with the Wizard® Genomic DNA Purification Kit as indicated by the manufacturer (Promega, Madison, WI). The specific SRY DNA was then amplified from extracted kidney DNA using PCR System 2400 with the following primers (22):

Forward: 5'-AAGCGCCCCATGAATGC-3'

Reverse: 5'-AGCCAACTTGCGCCTCTCT-3'

Genotyping: Identification of wild type and mutated *Pkhd1* genes in the PCK rats was performed via PCR (as above) using the primers specified by Charles River:

Mut-Forward: 5'-AAG CCA AAT CTT TCT CTT TTC CT-3'

Mut-Reverse: 5'- CTT GCT GTC CGA ATA CCA C -3'

Wild type-Forward:5'-ACT GCC TTT TAC TGA AGC ATT TAA C-3'

Wild type-Reverse: 5'- TGG AAG GAA AAG TTG CCC T -3'

Exosome studies: Primary renal tubule cells from normal Sprague Dawley rats were isolated as above. After 2 days in culture, S1 medium with exosome-free fetal calf serum was used. Two days later, the cell culture supernatant was centrifuged at 300g for 10 minutes to remove cells, 2000g x 10 minutes to remove dead cells, 10,000g x 30 minutes to remove cells debris. The resultant supernatant is centrifuged at 100,000g x 70 minutes, washed and centrifuged again at 100,000g x 70 minutes to obtain exosomes. After fixation in 2% paraformaldehyde/2% glutaraldehyde/0.1M phosphate buffer and dehydration in graded ethyl alcohols (70-100%), exosome isolation was verified by electron microscopy (Tecnai G2 12 Bio Twin microscope, FEI, Hillsboro, OR). Prior to addition to cell culture, exosome RNA was labeled with red fluorescent dye and exosome protein with green fluorescent dye via exo-glow (SBI, Mountain View, CA) according to the supplier's protocol. PCK tubular cells were isolated by collagenase digestion and cultured as for Sprague Dawley cells (above). When the cells were 50-70% confluent, the medium was changed to S1 medium with 10% exosome free fetal calf serum and fluorescently labeled exosomes (10 μ protein/10⁶ cells) added to the cells and imaging performed approximately 16 hours later. In separate studies, exosome treated cells were cultured for 2 days prior to resuspension in matrigel (BD Biosciences, Bedford, MA) at a concentration of 3000 cells/ml and incubated in glass bottom dishes. In some studies, PCK and SD cells were cultured together in the following proportions:

PCK	100%	98%	90%	80%	0%
SD	0%	2%	10%	20%	100%

Cyst number was quantified in blinded images at 4 days. At 7 days, the cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton and incubated with rhodamine phalloidin and Hoescht 3342 (both Life Technologies, Carlsbad, CA). Images were collected using an

Olympus confocal microscope equipped for two photon imaging. Z-stacks were collected to evaluate cyst formation in 3D.

Immunoblotting. Exosome samples and fibrocystin protein control (Santa Cruz Biotechnology, Santa Cruz, CA) were fractionated by electrophoresis through 16.5% polyacrylamide Tris-tricine gels. After transfer and blocking, blots were incubated with anti-fibrocystin or anti-CD63 (Santa Cruz).

Statistics. Data are expressed as means \pm 1 standard error. Analysis of variance was used to determine if differences among mean values reached statistical significance. Student's t test (2 tailed, 2 sample, unequal variance) was used for comparisons between groups (GraphPad Prism, LaJolla, CA). Tukey's test was used to correct for multiple comparisons. The null hypothesis was rejected at $p < 0.05$.

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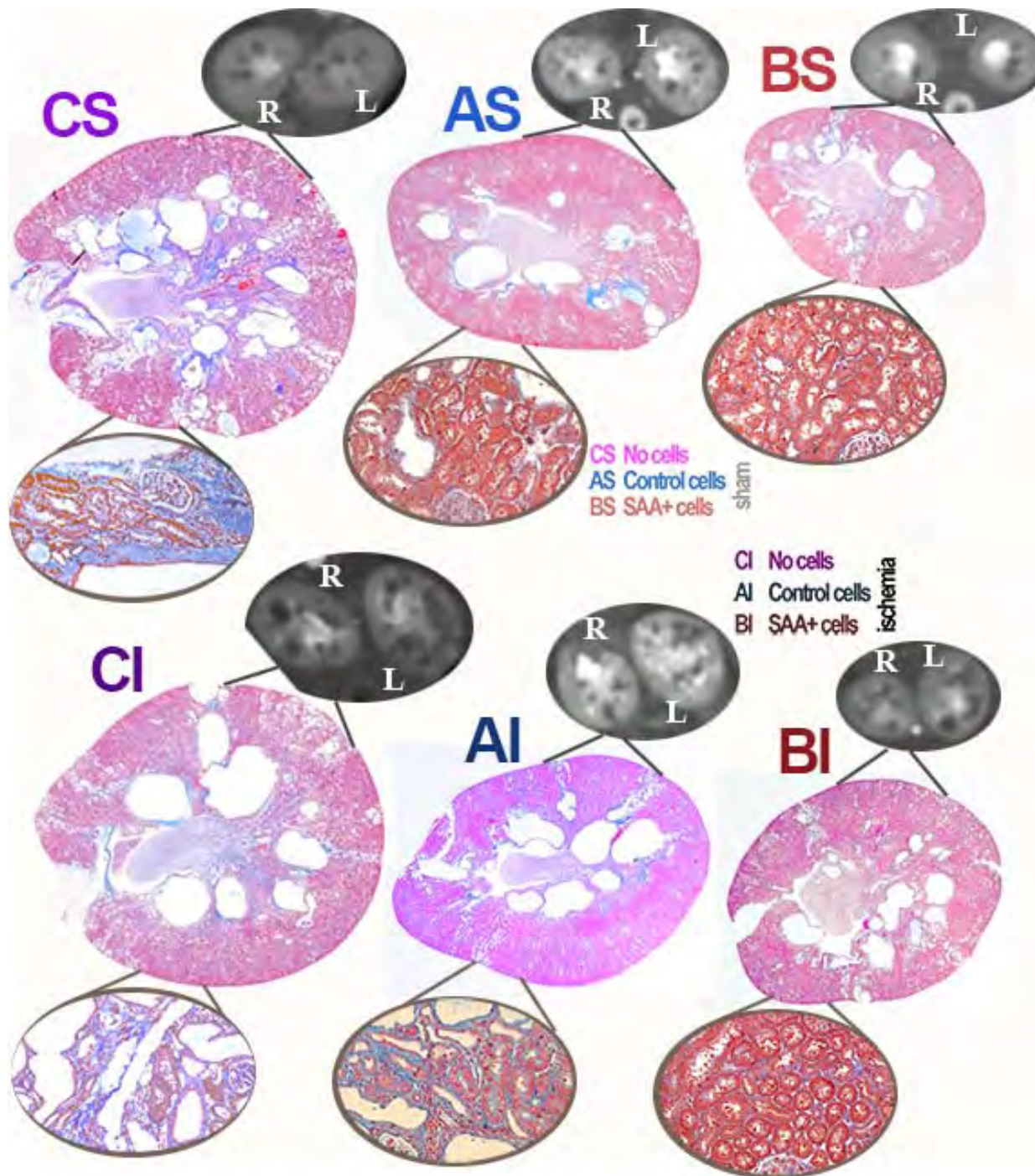
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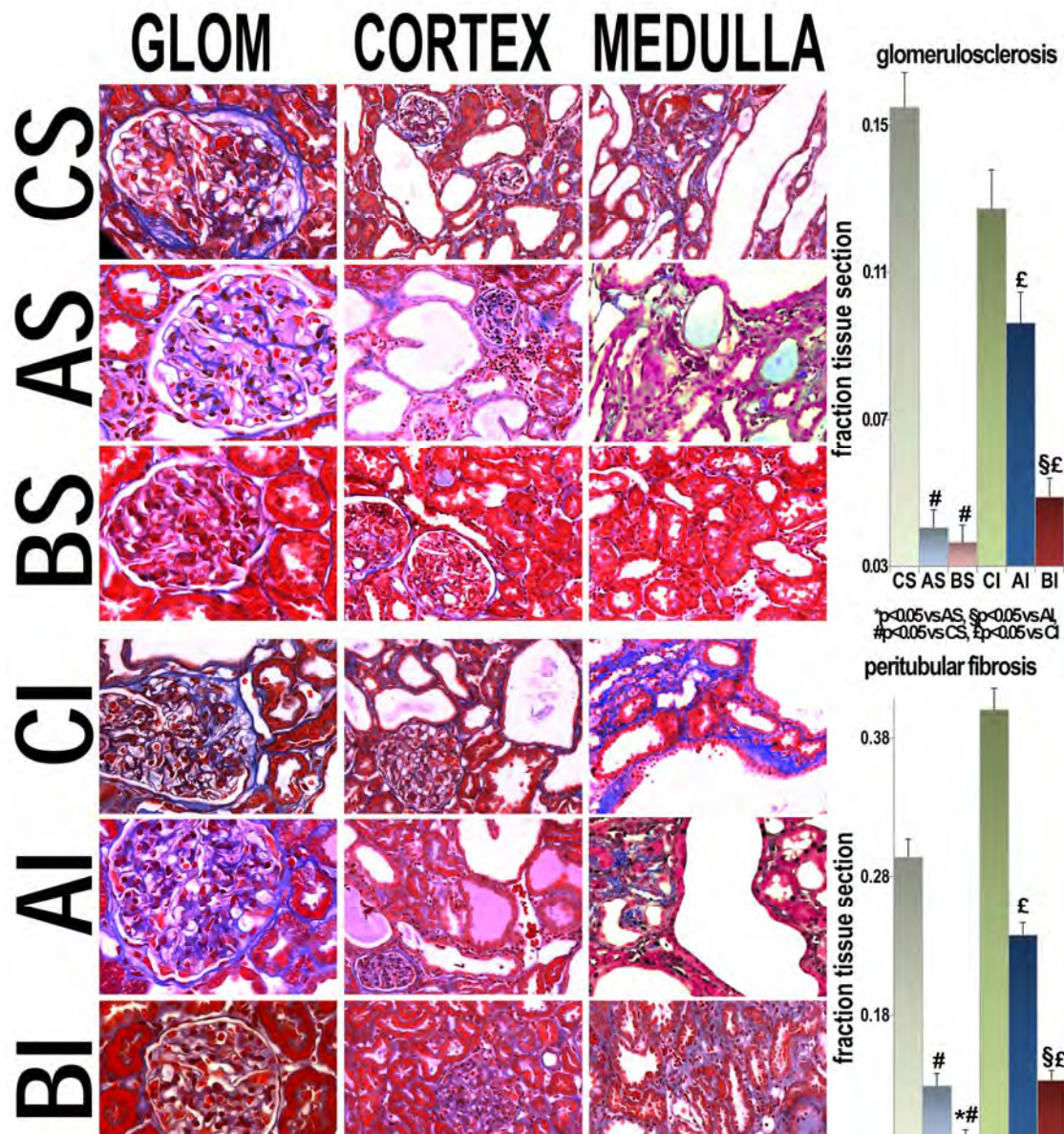
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FIGURE1



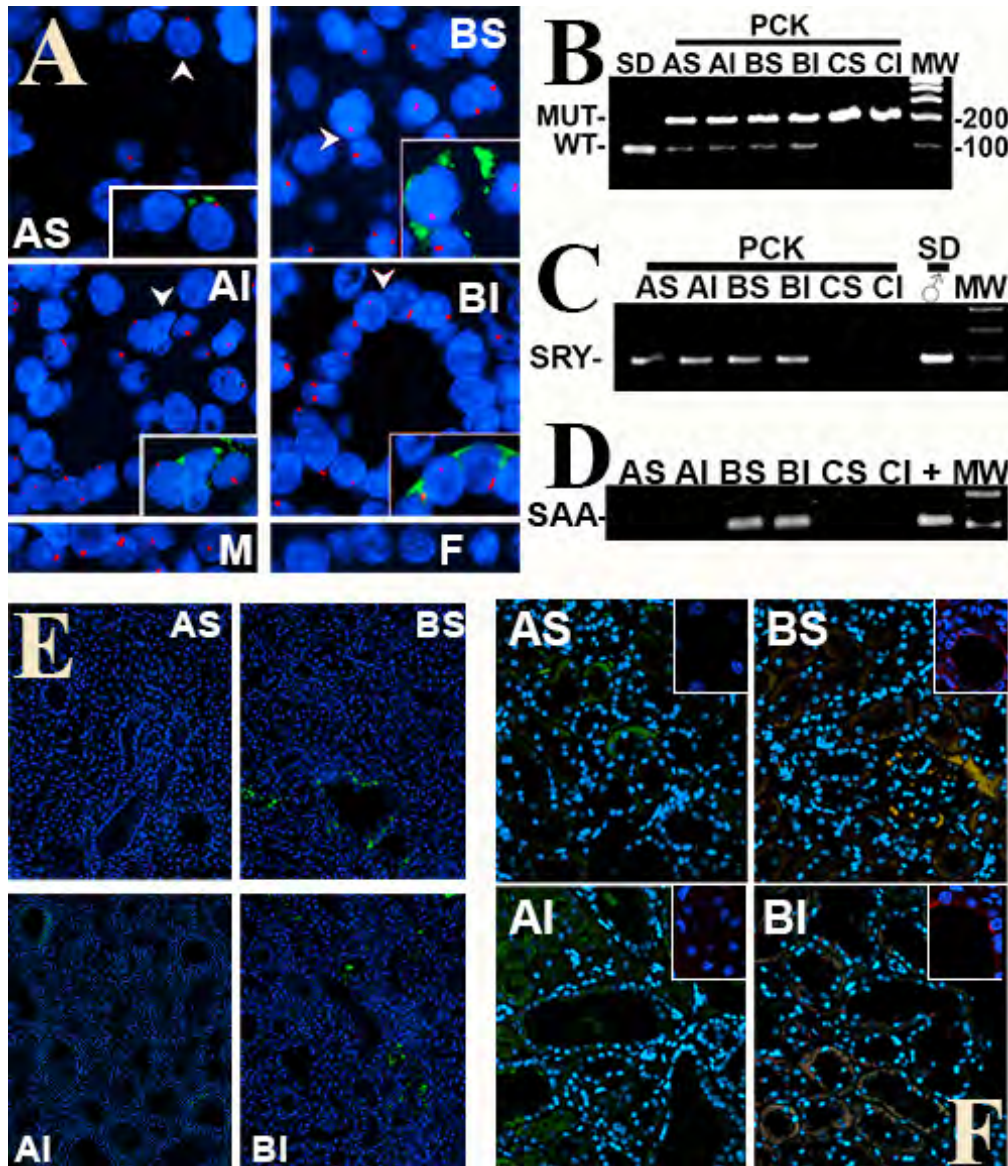
The power of cytotherapy. When compared to no cell transplant (C) groups, treatment of PCK rats with SAA+ (B) or control (A) cells improves cyst volume and structure at 25 weeks of age. The termination point was 15 weeks after the final cell transplant. Representative dynamic contrast CT images and PAS stained and trichrome stained sections (insets) are presented. In this figures and subsequent figures, CS=no cells/sham surgery; AS=A(control, SAA-)cells/sham surgery; BS=B(SAA+)cells/sham surgery; CI=no cells/renal ischemia; AI=A(control, SAA-)cells/renal ischemia; BI=B (SAA+)cells/renal ischemia.

FIGURE 2



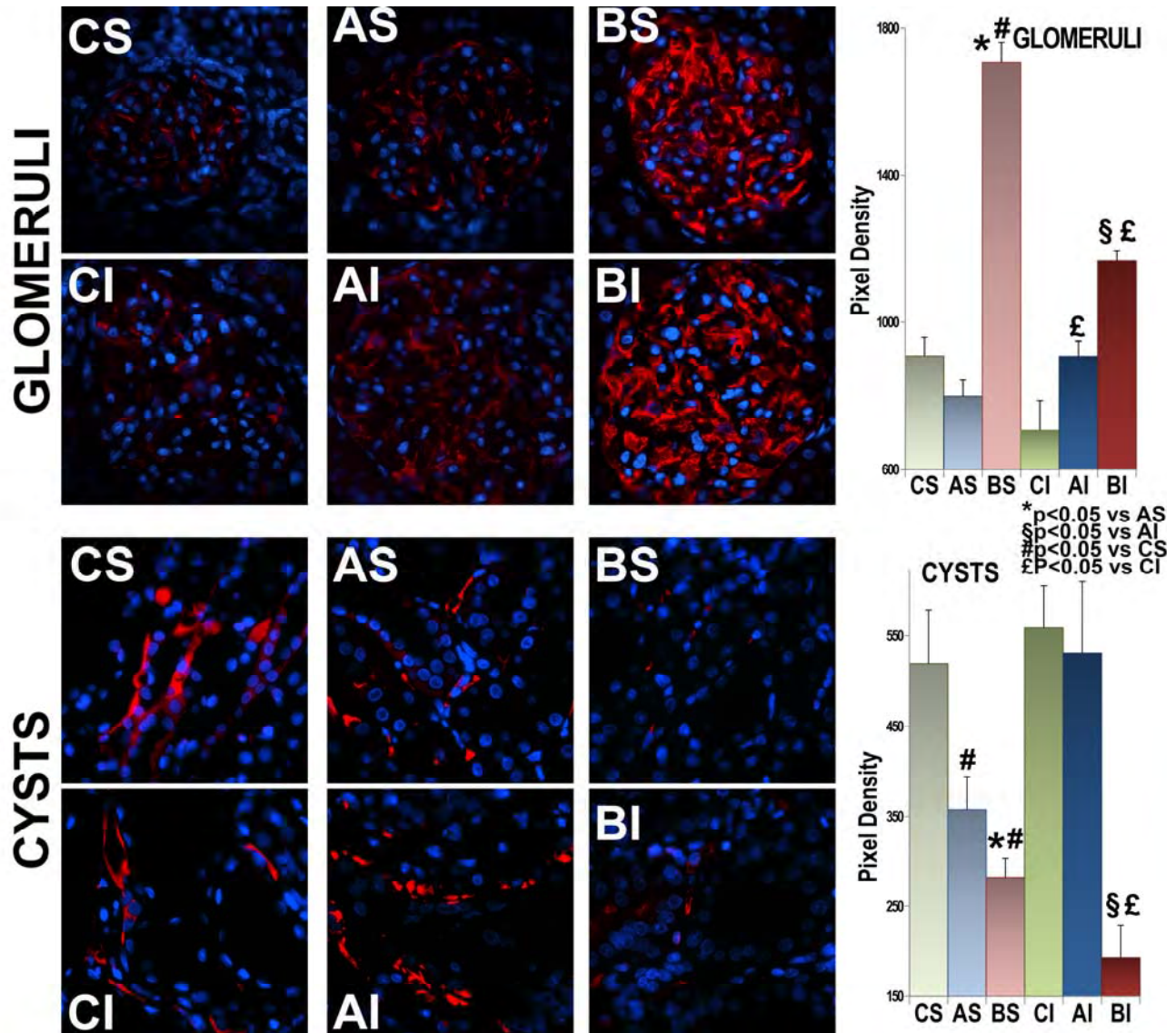
Histology. Representative trichrome stained sections (resulting in blue labeling of fibrous tissue) of glomeruli (glom), cortex and medulla in each of the 6 groups are presented. Quantification of glomerulosclerosis (in a total of 5602 glomeruli) and peritubular fibrosis (in 4249 microscope fields) is presented in the graphs. Glom, glomeruli; *p<0.05 vs AS; §p<0.05 vs AI; #p<0.05 vs CS; £p<0.05 vs CI; other abbreviations as in figure 1.

FIGURE 3



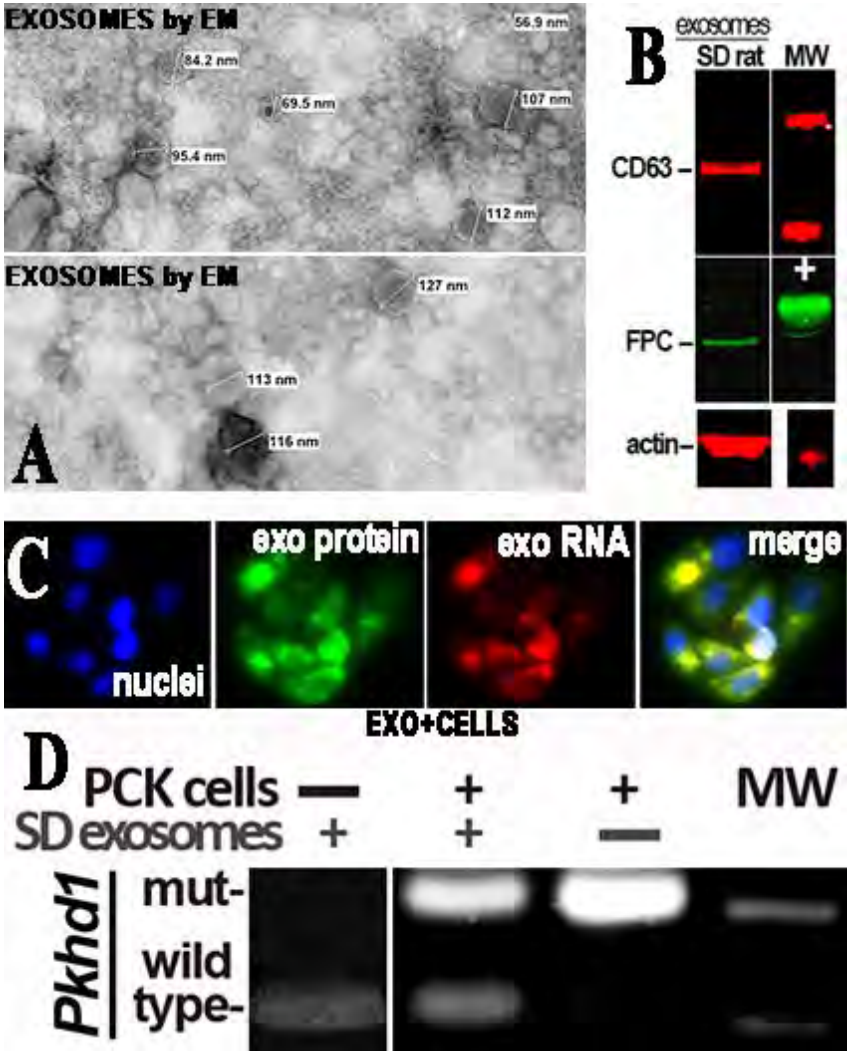
Engraftment of transplanted cells: Multiple methods were employed to demonstrate the persistence of infused cells in transplanted kidneys. (A) Fluorescence in situ hybridization (FISH) showed Y chromosome + (red) nuclei in kidneys of female rats that received male cells. The insets show green fluorescence of areas at arrows, demonstrating GFP and the Y chromosome in the same cells. Nuclei in this panel and panels E and F are stained with DAPI (blue). (B) Genotyping by PCR demonstrated the presence of both wild type (WT) and mutated (MUT) *Pkhd1* transcripts in kidneys from PCK rats that received SAA+ or SAA- cells but not in PCK rats that received no cells. Only the wild type transcript is present in normal Sprague Dawley (SD) rats. (C) PCR for the SRY male determining gene showed similar results: SRY was present in female AS, AI, BS, BI transplanted with male cells and male (♂) SD but not female CS or CI rats. (D) mRNA for SAA is present only in BS and BI kidneys demonstrating transcriptional activity of the SAA gene from donor B cells. pcDNA3.3-SAA1 was used as the positive (+) control for SAA PCR. (E) GFP positive cells are also found in transplanted kidneys. (F) Immunostaining for SAA (red) demonstrates co-localization (orange) with GFP in BS and BI groups. The insets in F show higher power confocal images for SAA (red) in cyst epithelium. Animal group labels are as in figure 1. M=normal male; F=normal male; MW=molecular weight markers.

FIGURE 4



Protection of microvasculature with cytotherapy. Representative images stained for CD31 (platelet endothelial cell adhesion molecule [PECAM], red) show better preserved glomerular vasculature in SAA+ B cell groups. In addition, less vasculature surrounding abnormal cystic epithelium is seen in the groups that received SAA+ B cells. Quantification of red pixel density representing CD31 staining in 266 images is shown in the graphs. Abbreviations are as in figure 1.

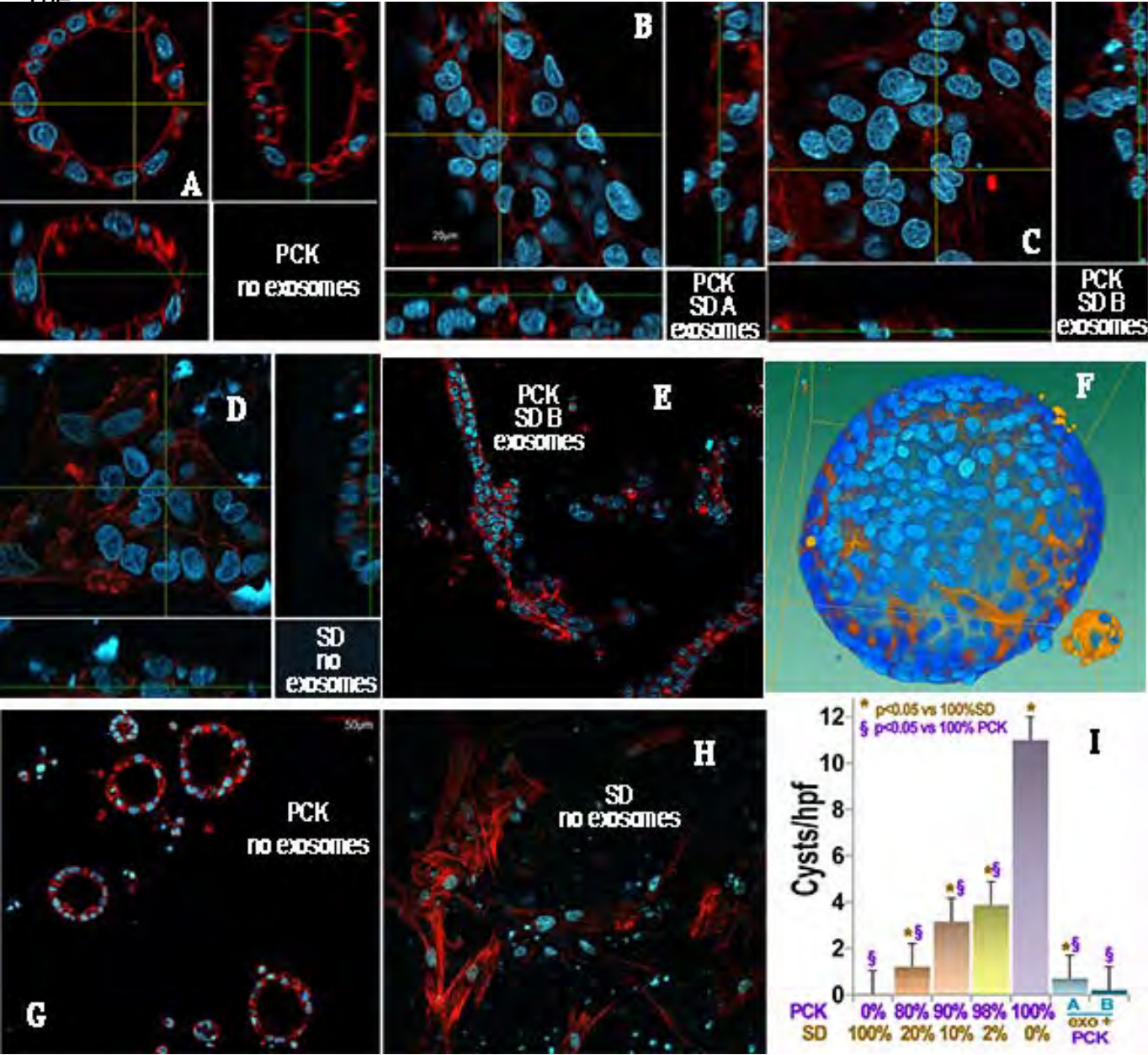
FIGURE 5



Exosome Uptake by PCK cells. Isolation of exosomes is confirmed by electron microscopy (EM, A) and expression of CD63 (B). Exosomes from normal Sprague Dawley rats express normal fibrocystin (FPC, B) Internal exosome protein was fluorescently labeled green and RNA red prior to incubation of PCK cells with the exosomes for 16 hours. Panel C shows uptake of the exosomes by the primary PCK renal cells. PCR genotyping demonstrates expression of wild type and mutant *Pkhd1* in the PCK cells exposed to exosomes (D). Wild type *Pkhd1* is found in SD exosomes and only mutant *Pkhd1* in control PCK cells.

FIGURE 6

The



effect of exosomes on PCK cysts in culture. Representative confocal images of cells stained with rhodamine phalloidin to label actin red and Hoescht to label nuclei blue demonstrate cysts in tubule cells from PCK rats grown in matrigel without exosome treatment (A, F, G). Neither PCK cells cultured with exosomes (B, C, E) from Sprague Dawley (SD) rats nor SD tubular cells (D, H) form cysts in matrigel. The higher power images (A-D) show orthogonal projections to demonstrate the clear presence or absence of cysts. Panel F shows a 3D reconstruction of multiple image also demonstrate the cystic nature of the structures formed by PCK cells. The lower power images (E, G, H) demonstrate tubular structures in the presence of exosomes, multiple cysts in one field of PCK cells and the absence of cysts in SD cells, respectively. Quantification of cyst number in a total of 168 fields is presented in the

graph (I). B exosomes are from SAA+ cells, A are from SAA- control cells. Both A and B exosomes contain wild type *Pkhd1* (above).

Table 1. Kidney Functional and Structural Measures in Treated and Control PCK rats

	CS ^a	AS	BS	CI	AI	BI
Cyst volume (ml)	0.5±0.01	0.4±0.03 [#]	0.3±0.06 ^{bd}	0.7±0.01	0.6±0.05	0.4±0.04 ^{ce}
Albuminuria (g/g creat)	4.6±0.6	1.9±0.1 [#]	1.3±0.1 ^b	4.3±0.2	2.4±0.2 ^f	1.4±0.1 ^{ce}
BUN (mg/dl)	35±2.3	24±0.7 [#]	19±0.6 ^{bd}	36±0.9	29±1.5 ^f	23±0.3 ^{ce}
Kidney weight (g)	2.8±0.1	2.6±0.1 [#]	2.2±0.1 ^{bd}	3.9±0.6	3.1±0.1	2.8±0.1 ^{ce}

^aanimal groups: CS, control (no cells)/sham surgery; AS, A (control) cells/sham surgery; BS, B (SAA+) cells/sham surgery; CI, control (no cells)/ischemia; AI, A (control) cells/ischemia; BI, B (SAA+) cells/ischemia

^bp<0.05 vs AS ^cp<0.05 vs AI ^dp<0.05 vs CS ^ep<0.05 vs CI